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(54) Title: METHOD FOR MODIFYING LIGNIN COMPOSITION AND INCREASING IN VIVO DIGESTIBILITY OF FORAGES

(57) Abstract: Methods for transforming forage legumes or woody plants with a DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof in either a sense or antisense orientation under a lignification-associated tissue specific promoter have been found, resulting in the down-regulation of the corresponding homologous gene either through antisense inhibition or sense suppression, as well as reduced lignin content and modified lignin composition in the transgenic plants. The expression of the caffeoyl CoA 3-O-methyltransferase transgene produces an increased syringyl lignin to guaiacyl lignin ratio in the transformed plant, and greatly improved forage digestibility.

METHOD FOR MODIFYING LIGNIN COMPOSITION AND INCREASING IN VIVO DIGESTIBILITY OF FORAGES

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisonal Application No. 60/192,086 filed March 24, 2000.

TECHNICAL FIELD OF INVENTION

5   This invention relates to a method of transforming plants, transformed plants and use thereof.

BACKGROUND OF THE INVENTION

Lignin is the major structural component of secondarily thickened plant cell walls. It is a complex polymer of hydroxylated and methoxylated phenylpropane units, linked via

10   oxidative coupling that is probably catalyzed by both peroxidases and laccases (Boudet, et al. 1995. "Tansley review No. 80: Biochemistry and molecular biology of lignification," *New Phytologist* 129:203-236). Lignin imparts mechanical strength to stems and trunks, and hydrophobicity to water-conducting vascular elements. Although the basic enzymology of lignin biosynthesis is reasonably well understood, the regulatory steps in

15   lignin biosynthesis and deposition remain to be defined (Davin, L.B. and Lewis, N.G. 1992. "Phenylpropanoid metabolism: biosynthesis of monolignols, lignans and neolignans, lignins and suberins," *Rec Adv Phytochem* 26:325-375).

There is considerable interest in the potential for genetic manipulation of lignin levels and/or composition to help improve digestibility of forages and pulping properties of

20   trees (Dixon, et al. 1994. "Genetic manipulation of lignin and phenylpropanoid compounds involved in interactions with microorganisms," *Rec Adv Phytochem* 28:153178; Tabe, et al. 1993. "Genetic engineering of grain and pasture legumes for improved nutritive value," *Genetica* 90:181-200; Whetten, R. and Sederoff, R. 1991. "Genetic engineering of wood," *Forest Ecology and Management* 43:301-316). Small decreases in lignin content have

25   been reported to positively impact the digestibility of forages (Casler, M.D. 1987. "In vitro digestibility of dry matter and cell wall constituents of smooth bromegrass forage," *Crop*

Sci 27:931-934). By improving the digestibility of forages, higher profitability can be achieved in cattle and related industries. In forestry, chemical treatments necessary for the removal of lignin from trees are costly and potentially polluting.

Lignins contain three major monomer species, termed p-hydroxyphenyl (H),

5   guaiacyl (G) and syringyl (S), produced by reduction of CoA thioesters of coumaric, ferulic and sinapic acids, respectively (see FIG. 1). In angiosperms, guaiacyl and syringyl units predominate, and the S/G ratio affects the physical properties of the lignin. The S and G units are linked through five different dimer bonding patterns (Davin, L.B. and Lewis, N.G. 1992. *Rec Adv Phytochem* 26:325-375). The mechanisms that determine the

10   relative proportions of these linkage types in a particular lignin polymer are currently unknown. Furthermore, there is considerable debate as to whether lignin composition and structure are tightly controlled, or are flexible depending upon monomer availability (Lewis, N.G. 1999. "A 20th century roller coaster ride: a short account of lignification," *Current Opinion in Plant Biology* 2:153-162; Sederoff, et al. 1999, "Unexpected variation

15   in lignin," *Current Opinion in Plant Biology* 2:145-152).

Lignin levels increase with progressive maturity in stems of forage crops, including legumes such as alfalfa (Jung, H.G. and Vogel, K.P. 1986. "Influence of lignin on digestibility of forage cell wall material," *J Anim Sci* 62:1703-1712) and in grasses such as tall fescue (Buxton, D.R. and Russell, J.R. 1988. "Lignin constituents and cell wall

20   digestibility of grass and legume stems," *Crop Sci* 28:553-558). In addition, lignin composition changes with advanced maturity towards a progressively higher S/G ratio (Buxton, D.R. and Russell, J.R. 1988. *Crop Sci* 28:553-558). Both lignin concentration (Albrecht, et al. 1987. "Cell-wall composition and digestibility of alfalfa stems and leaves," *Crop Sci* 27:735-741; Casler, M.D. 1987. *Crop Sci* 27:931-934; Jung, H.G. and Vogel,

25   K.P. 1986. *J Anim Sci* 62:1703-1712) and lignin methoxyl content, reflecting increased S/G ratio (Sewalt, et al. 1996. "Lignin impact on fiber degradation. 1. Quinone methide intermediates formed from lignin during *in vitro* fermentation of corn stover," *J Sci Food Agric* 71:195-203), have been reported to negatively correlate with forage digestibility for ruminant animals. Although a number of studies have linked decreased forage digestibility

30   to increased S/G ratio as a function of increased maturity (Buxton, D.R. and Russell, J.R. 1988. *Crop Sci* 28:553-558; Grabber, et al. 1992. "Digestion kinetics of parenchyma and

sclerenchyma cell walls isolated from orchardgrass and switchgrass," *Crop Sci* 32: 806-810), other studies have questioned the effect of lignin composition on digestibility (Grabber, et al. 1997. "p-hydroxyphenyl, guaiacyl, and syringyl lignins have similar inhibitory effects on wall degradability," *J Agric Food Chem* 45:2530-2532). Further, the

5 hardwood gymnosperm lignins are highly condensed, essentially lacking S residues, and this makes them less amenable to chemical pulping, in apparent contradiction to the concept that reducing S/G ratio would be beneficial for forage digestibility. The reported lack of agreement in the relationship of lignin composition to forage digestibility and chemical pulping is partly due to the fact that the studies to date either have been in vitro,

10 or have compared plant materials at different developmental stages, different varieties or even different species. Therefore, the development of isogenic lines that can be directly compared to reveal the effects of altered S/G ratio on forage digestibility would be beneficial.

The formation of the G and S units of lignin requires the activity of O-methyl-

15 transferase enzymes. In angiosperms, the caffeic acid 3-O-methyltransferase (COMT) of lignin biosynthesis was originally described as being bifunctional, converting caffeic acid to ferulic acid and converting 5-hydroxyferulic acid to sinapic acid (Davin, L.B. and Lewis, N.G. 1992. *Rec Adv Phytochem* 26:325-375), as shown in FIG. 1. Methylation of the caffeate moiety also occurs at the level of the CoA thioester, catalyzed by caffeoyl CoA

20 3-O-methyltransferase (CCOMT) (Pakusch, et al., 1989, "S-adenosyl-L-methionine: trans-caffeoyl-coenzyme A 3-O-methyltransferase from elicitor-treated parsley cell suspension cultures," *Arch Biochem Biophys* 271:488-494). The involvement of the CCOMT enzyme in a parallel pathway to lignin monomer formation has been proposed (Ye, et al. 1994. "An alternative methylation pathway in lignin biosynthesis in *Zinnia*," *Plant Cell* 6:1427-1439;

25 Zhong, et al. 1998. "Dual methylation pathways in lignin biosynthesis," *Plant Cell* 10:2033-2045). In vivo labeling studies in *Magnolia kobus* have shown that the methylation status of lignin monomers can also be determined at the level of the aldehyde or alcohol (Chen, et al. 1999. "Evidence for a novel biosynthetic pathway that regulates the ratio of syringyl to guaiacyl residues in lignin in the differentiating xylem of *Magnolia*

30 *kobus* DC," *Planta* 207:597-603). This is supported by the observation that the enzyme designated as ferulate 5-hydroxylase has a higher affinity for feruloyl aldehyde than for

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ferulic acid, at least in sweet gum (Osakabe, et al. 1999. "Coniferyl aldehyde 5-
hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms," *Proc
Natl Acad Sci USA* 96:8955-8960) and *Arabidopsis* (Humphreys, et al. 1999. "New routes
for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-

5   hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase," *Proc Natl
Acad Sci USA* 96:10045-10050). Furthermore, 5-hydroxyconiferyl aldehyde has recently
been shown to be a good substrate for COMT from various tree species (Li, et al. 2000. "5-
Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol
formation, a new view of monolignol biosynthesis in angiosperms," *J Biol Chem*

10   275:6537-6545). It has been reported that the inhibitory effect of 5-hydroxyconiferyl
aldehyde on methylation of caffeate by COMT might prevent COMT from carrying out the
first methylation step in the biosynthesis of S lignin (Li, et al. 2000. *J Biol Chem*
275:6537-6545). Thus, although studies of enzyme substrate specificities in vitro suggest
that lignin monomers can be formed via the operation of a complex metabolic grid,

15   involving O-methylation at multiple stages as shown in FIG. 1, whether this occurs in vivo
has yet to be determined.

Several studies have addressed the properties of the O-methyltransferases involved
in lignin biosynthesis in the world's major forage legume, alfalfa *(Medicago sativa* L.)
(Gowri, et al. 1991. "Stress responses in alfalfa *(Medicago sativa* L.) X. Molecular cloning

20   and expression of S-adenosyl-L-methionine: caffeic acid 3-O-methyltransferase, a key
enzyme of lignin biosynthesis," *Plant Physiol* 97:7-14; Inoue, et al. 1998. "Developmental
expression and substrate specificities of alfalfa caffeic acid 3-O-methyltransferase and
caffeoyl CoA 3-O-methyltransferase in relation to lignification," *Plant Physiol* 117:761-
770; Kersey, et al. 1999. "Immunolocalization of two lignin O-methyltransferases in stems

25   of alfalfa *(Medicago sativa* L.)," *Protoplasma* 209:46-57). COMT from alfalfa expressed
in *E. coli* shows preference (approximately 2:1) for 5-hydroxyferulic acid over caffeic acid,
whereas CCOMT shows a similar degree of preference for caffeoyl CoA compared to 5-
hydroxyferuolyl CoA (Inoue, et al. 1998. *Plant Physiol* 117:761-770). These studies
suggest, but do not prove, that COMT may be involved preferentially in the formation of S

30   lignin in alfalfa, and CCOMT in the formation of G lignin.

The substrate preference of COMT in crude alfalfa stem extracts changes with increasing internode maturity, in a manner consistent with the increase in lignin methoxyl group content with increasing maturity (Inoue, et al. 1998. *Plant Physiol* 117:761-770; Inoue, et al. 2000. "Substrate preferences of caffeic acid/5-hydroxyferulic acid 3-O-

5   methyltransferases in developing stems of alfalfa *(Medicago sativa L.)*," *Arch Biochem Biophys* 375:175-182). Thus, in young internodes, the activity shows a preference for caffeic acid over 5-hydroxyferulic acid, whereas the opposite is true in the older internodes. An O-methyltransferase with preference for caffeic acid (COMT II) has recently been separated from the previously characterized COMT, and does not react with

10   antisera recognizing the products of the previously characterized alfalfa COMT or CCOMT genes. This enzyme is most active against caffeic acid, for which it has a very low Km value (approximately 40-fold lower than lignification-associated COMT), but also methylates 5-hydroxyferulic acid, caffeoyl CoA, 5-hydroxyferuolyl CoA, quercetin and catechol (Inoue, et al. 2000. *Arch Biochem Biophys* 375:175-182). It is only present in

15   young internodes and has disappeared by the fifth internode.

Tissue print hybridization analysis indicates that both COMT and CCOMT transcripts are localized to developing xylem elements in alfalfa stems, whereas CCOMT transcripts are also found in phloem (Inoue, et al. 1998. *Plant Physiology* 117:761-770). Immunolocalization studies at the light and electron microscope levels demonstrated

20   expression of both COMT and CCOMT in the cytoplasm of alfalfa xylem parenchyma cells (Kersey, et al. 1999 *Protoplasma* 209:46-57). The presence of both enzymes in the same cells is consistent with the "metabolic grid" hypothesis for lignin monomer formation.

There have been several reports on the effects of down-regulation of COMT

25   activity on lignin content and composition in transgenic tobacco and poplar (Ni, et al. 1994. "Reduced lignin in transgenic plants containing an engineered caffeic acid O-methyltransferase antisense gene," *Transgenic Res* 3:120-126; Atanassova, et al. 1995. "Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation," *Plant J* 8:465-477; Van Doorsselaere, et al.

30   1995. "A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid O-methyltransferase activity," *Plant J* 8:855-864; Zhong, et al. 1998. *Plant Cell* 10:2033-

2045). The results of these studies have been somewhat contradictory, possibly due to

unspecified differences in tissue maturity, use of homologous versus heterologous

transgenes, and use of different methods for lignin analysis. However, in cases where

COMT has been reduced to levels below approximately 20% of wild-type by expression of

5   a homologous transgene, a strong reduction in S/G ratio is accompanied by no apparent

change in lignin content (Atanassova, et al. 1995. *Plant J* 8:465-477; Van Doorsselaere, et

al. 1995. *Plant J* 8:855-864). In tobacco, down-regulation of CCOMT leads to a

corresponding decrease in Klason lignin levels accompanied by decreases in the absolute

levels of both S and G units (Zhong, et al. 1998. *Plant Cell* 10:2033-2045).

10   Most studies on genetic modification of lignin biosynthesis in transgenic plants

have utilized the cauliflower mosaic virus 35S promoter to drive expression of sense or

antisense lignification-associated genes (Halpin, et al. 1994. "Manipulation of lignin

quality by down-regulation of cinnamyl alcohol dehydrogenase," *Plant J* 6:339-350; Ni, et

al. 1994. *Transgenic Res* 3:120-126; Atanassova, et al. 1995. *Plant J* 8:465-477; Van

15   Doorsselaere, et al. 1995. *Plant J* 8:855-864; Piquemal, et al. 1998. "Down-regulation of

cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic

tobacco plants," *Plant J* 13:71-83; Zhong, et al. 1998. *Plant Cell* 10:2033-2045; Baucher,

et al. 1999, "Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa

(*Medicago sativa* L.) and the effect on lignin composition and digestibility," *Plant Mol*

20   *Biol* 39:437-447). However, more effective down-regulation may be obtained by driving

expression of the transgene by a vascular-tissue specific promoter. For example,

modification of lignin composition by overexpression of ferulate 5-hydroxylase in

transgenic Arabidopsis was more effective if the transgene was driven by the lignification-

associated Arabidopsis cinnamate 4-hydroxylase promoter than by the constitutive 35S

25   promoter (Meyer, et al. 1998. "Lignin monomer composition is determined by the

expression of a cytochrome P450-dependent monooxygenase in Arabidopsis," *Proc Natl*

Acad Sci USA 95:6619-6623).

            To date, there have been very few published reports on the genetic modification of

lignin in forage crops, and most studies having concentrated on model systems such as

30   Arabidopsis and tobacco, or tree species such a poplar. In one study, down-regulation of

cinnamyl alcohol dehydrogenase, an enzyme later in the monolignol pathway than COMT

or CCOMT, led to a small but significant improvement in in vitro dry matter digestibility in transgenic alfalfa (Baucher, et al. 1999. *Plant Mol Biol* 39:437-447). U.S. Patent No. 5,451,514 discloses a method of altering the content or composition of lignin in a plant by stably incorporating into the genome of the plant a recombinant DNA encoding an mRNA

5   having sequence similarity to cinnamyl alcohol dehydrogenase. U.S. Patent No. 5,850,020 discloses a method for modulating lignin content or composition by transforming a plant cell with a DNA construct with at least one open reading frame coding for a functional portion of one of several enzymes isolated from *Pinus radiata* (pine) or a sequence having 99% homology to the isolated gene: cinnamate 4-hydroxylase (C4H), coumarate 3-

10   hydroxylase (C3H), phenolase (PNL), O-methyltransferase (OMT), cinnamoyl-CoA reductase (CCR), phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (4CL), and peroxidase (POX). U.S. Patent No. 5,922,928 discloses a method of transforming and regenerating Populus species to alter the lignin content and composition using an O-methyltransferase gene. The question of how altering S/G ratio might affect digestibility

15   of forage species is still unanswered.

It has now been found that transformation of plants with the lignin biosynthetic enzyme genes COMT or CCOMT in either a sense or antisense orientation under a lignification-associated tissue specific promoter, leading to the down-regulation of the corresponding homologous gene as well as reduced lignin content and modified lignin

20   composition in the transgenic plants, results in significant improvements in forage digestibility, particularly in the case of CCOMT down-regulation.

SUMMARY OF THE INVENTION

In one aspect, the present invention is a method for modulating the lignin content of a forage legume comprising transforming a forage legume cell with a vector comprising a

25   lignification-associated tissue specific promoter functionally linked to a DNA construct comprising at least one open reading frame encoding for either a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof; and generating plants from the transformed forage legume cell. In another embodiment, the forage legume cell is co-transformed with

30   one vector comprising a lignification-associated tissue specific promoter functionally linked to a DNA construct comprising at least one open reading frame encoding for a

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caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof and another vector comprising a lignification-associated tissue specific promoter functionally linked to a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof. In yet another

5 embodiment, a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated

10 tissue specific promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2* promoter.

In another aspect, the present invention is a method for producing a forage legume

15 having altered lignin composition comprising transforming a forage legume cell with a DNA construct comprising either at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and cultivating

20 said transgenic cell under conditions conducive to regeneration and plant growth. In another embodiment, the forage legume cell is co-transformed with one DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter and another DNA construct comprising at least one

25 open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter. In yet another embodiment, a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue

30 specific promoter and at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of

a second lignification-associated tissue specific promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2*

5 promoter.

In another aspect, the present invention is a method for improving the digestibility of forage legumes comprising stably incorporating into the genome of said forage legume a DNA construct comprising at least one open reading frame encoding for a 3-O-methyltransferase enzyme or a fragment thereof from the lignin biosynthetic pathway

10 under expression control of a lignification-associated tissue specific promoter, wherein expression of the enzyme or enzyme fragment produces a change in lignin composition in the forage legume. One enzyme useful in this method is caffeoyl CoA 3-O-methyltransferase, which preferably causes a reduction in guaiacyl lignin content. In another embodiment, the forage legume is co-transformed with one DNA construct

15 comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter and another DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue

20 specific promoter. In yet another embodiment, a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of

25 a second lignification-associated tissue specific promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2* promoter.

30 In another aspect, the present invention is a method for producing a woody plant having altered lignin composition comprising transforming a woody plant cell with a DNA

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construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeoyl CoA 3-O-methyltransferase enzyme or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and cultivating the transgenic

5   cell under conditions conducive to regeneration and plant growth. In another embodiment, the woody plant cell is co-transformed with one DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter and another DNA construct comprising at least one open reading frame encoding

10   for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter. In yet another embodiment, a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at

15   least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated tissue specific promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense

20   orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2* promoter.

In another aspect, the present invention is a method for modulating the lignin content of a woody plant comprising transforming a woody plant cell with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa*

25   caffeoyl CoA 3-O-methyltransferase enzyme or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and cultivating the transgenic cell under conditions conducive to regeneration and plant growth. In another embodiment, the woody plant cell is co-transformed with one DNA construct comprising at least one

30   open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific

promoter and another DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter. In yet another embodiment, a DNA construct comprising in tandem at least one open reading

5   frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated tissue specific promoter, wherein said first and second

10   lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2* promoter.

        In another aspect, the present invention is a method for making lignins with altered

15   dimer bonding patterns comprising transforming a plant cell with a DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or a caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and cultivating the transgenic cell under conditions conducive to

20   regeneration and plant growth. In another embodiment, the plant cell is co-transformed with one DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter and another DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-

25   O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter. In yet another embodiment, a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame

30   encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated tissue specific

promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different, can be used in this method. The open reading frame can be in either a sense orientation or an antisense orientation. An exemplary lignification-associated tissue specific promoter is a bean *PAL2* promoter.

5   In yet another aspect, the present invention is a plant transformed by any of the methods disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A and 1B depict proposed biochemical pathways to lignin monomers. The "metabolic grid" shown in this scheme incorporates the results of recent studies suggesting
10   previously unexpected substrate specificities for ferulate 5-hydroxylase (F5H) and COMT (Humphreys, et al. 1999. *Proc Natl Acad Sci USA* 96:10045-10050; Osakabe, et al. 1999 *Proc Natl Acad Sci USA* 96:8955-8960; Li, et al. 2000. *J Biol Chem* 275:6537-6545).

FIG. 2 depicts binary constructs used for genetic modification of COMT and CCOMT expression in transgenic alfalfa. *PAL2* is the bean phenylalanine ammonia-lyase
15   *PAL2* promoter from -183 to -1226 bp (Liang, et al. 1989. "Developmental and environmental regulation of a phenylalanine ammonia-lyase 13-glucuronidase gene fusion in transgenic tobacco plants."*Proc Natl Acad Sci USA* 86:9284-9288) and NOS, the nopaline synthase terminator. Directionality of COMT and CCOMT is indicated by the arrows relative to the direction of the *PAL2* promoter. Constructs containing both COMT
20   and CCOMT in sense or antisense orientations were made by duplication of the PAL2/COMT/NOS and PAL2/CCOMT/NOS cassettes, and therefore, each cDNA is under control of a separate *PAL2* promoter. Introduction of both transgenes into a single plant was also achieved by co-transformation (Irdani, et al. 1998. "Construction of a new vector conferring methotrexate resistance in Nicotiana tabacum plants," *Plant Mol Biol* 37:1079-
25   1084) with single COMT and CCOMT constructs. All constructs are in the binary vector pCAMBIA3300.

FIG. 3A-3J depict COMT or CCOMT activities in stem tissue of control lines and COMT and/or CCOMT transgenic lines. FIG. 3A shows COMT activity in control plants transformed with empty pCAMBIA3300 vector. FIG. 3B shows COMT activity in plants
30   transformed with COMT in the sense orientation ("SC"). FIG. 3C shows COMT activity in plants transformed with a construct containing both COMT and CCOMT in the sense

orientation ("DS"), or by co-transformation with individual antisense COMT and CCOMT constructs. FIG. 3D shows COMT activity in plants transformed with COMT in the antisense orientation ("AC"). FIG. 3E shows COMT activity in plants transformed with a construct containing both COMT and CCOMT in the antisense orientation ("DA"), or by

5   co-transformation with individual antisense COMT and CCOMT constructs. FIG. 3F shows CCOMT activity in control plants transformed with empty pCAMBIA3300 vector. FIG. 3G shows CCOMT activity in plants transformed with CCOMT in the sense orientation ("SCC"). FIG. 3H shows CCOMT activity in plants transformed with a construct containing both COMT and CCOMT in the sense orientation ("DS"), or by co-

10   transformation with individual antisense COMT and CCOMT constructs. FIG. 3I shows CCOMT activity in plants transformed with CCOMT in the antisense orientation ("ACC"). FIG. 3J shows CCOMT activity in plants transformed with a construct containing both COMT and CCOMT in the antisense orientation ("DA"), or by co-transformation with individual antisense COMT and CCOMT constructs. The bars represent the means (solid

15   lines) and standard deviations (dashed lines) of the respective control populations. Enzyme activities were determined in the 6th-9th internodes of stems of identical developmental stage.

       FIG. 4 depicts typical gas chromatographs showing thioacidolysis products from lignin samples of wild-type (WT), COMT-suppressed (SC5), and CCOMT-suppressed

20   (ACC305) alfalfa plants. G, S and 5-hydroxyguaiacyl (5OHG) units are marked. The peaks appear as doublets because of the formation of erythro and threo isomers of each degradation product.

       FIG. 5 depicts the full nucleotide sequence for the 1097 bp coding region of alfalfa COMT (nucleotides 31-1128 of GenBank Accession No. M63853).

25   FIG. 6 depicts the full nucleotide sequence for the 743 bp coding region of alfalfa CCOMT (nucleotides 36-779 of GenBank Accession No. U20736).

DETAILED DESCRIPTION

       Using the methods of the present invention, the lignin content and composition of a forage legume such as alfalfa can be modified. Forage legumes are transformed with

30   genes encoding O-methyltransferase (OMT) enzymes from the lignin biosynthetic pathway inserted in the sense or antisense orientations and under a lignification-associated tissue

specific promoter. This transformation method can result in a variety of outcomes: a down-regulation of the corresponding homologous OMT genes, gene silencing, reduced OMT activity levels, reduced lignin content, and modified lignin composition in transgenic plants, and increased digestibility of transgenic plant materials in ruminant animals.

5   Transforming forage legumes with OMT enzymes has now made it possible to produce plants having modified lignin content and composition for direct comparison of the effects of lignin content and/or composition on forage digestibility. A preferred embodiment of the invention includes genetically engineering forage varieties with modified lignin to increase forage digestibility in animals. In another embodiment, plants are modified to

10   alter lignins and improve pulping characteristics for the paper industry.

        Transformation methods of the present invention utilize binary constructs comprising DNA sequences encoding O-methyltransferase (OMT) enzymes from the lignin biosynthetic pathway, preferably in conjunction with a gene promoter sequence and a gene termination sequence. In the present invention, full or partial DNA sequences either

15   isolated from alfalfa or produced by recombinant means and encoding or partially encoding O-methyltransferase (OMT) enzymes, are used in the transformation process. Preferably, a full length alfalfa COMT or CCOMT cDNA sequence in the sense or antisense orientation is placed in a binary vector with the cDNA being driven by a lignification-associated promoter. Alternatively, constructs can contain tandem COMT

20   and CCOMT cDNAs in sense or antisense orientations, with each cDNA being driven independently by a lignification-associated promoter. While full length COMT and CCOMT cDNA sequences are preferred, a genomic DNA sequence or a cDNA sequence encoding a portion of COMT or CCOMT can be used in the present invention, provided that the DNA sequence is of sufficient length so as to encode a fragment of the enzyme

25   wherein the fragment is effective for causing antisense inhibition or gene silencing of OMT expression.

        To drive expression of transgenes in forage legumes, a lignification-associated promoter is utilized. Any lignification-associated promoter known in the art can be useful in the present invention. However, since COMT and CCOMT enzymes are expressed in

30   the xylem and phloem parenchyma in alfalfa, lignification-associated promoters selective for vascular tissue are preferred. The promoter gene sequence can be endogenous to the

target plant, or it can be exogenous provided that the promoter is functional in the target plant. A lignification-associated tissue specific promoter can be used to target the production of sense or antisense RNA in the tissue of interest. An exemplary gene promoter sequence for use in forage legumes is the bean (*Phaseolus vulgaris*) *PAL2*

5 promoter.

Many gene termination sequences known in the art are useful in the present invention. The gene termination sequence can be from the same gene as the gene promoter sequence or from a different gene. An exemplary gene terminator sequence is the 3' end of the nopaline synthase, or *nos*, gene.

10 The DNA constructs of the present invention can optionally contain any selection marker effective in plant cells as a means of detecting successful transformation. Exemplary selection markers include antibiotic or herbicide resistance genes. Preferred selectable markers include a neomycin phosphotransferase gene or phosphinothricin acetyl transferase (*bar*) gene. For example, a preferable selection marker is the *bar* gene

15 encoding phosphinothricin acetyl transferase which confers resistance to phosphinothricin-based herbicides.

Transformation methods of the present invention include any means known in the art by which forage legumes can be successfully transformed using the DNA constructs disclosed herein. *Agrobacterium*-mediated transformation by leaf disk or biolistic

20 techniques followed by regeneration through somatic embryogenesis, direct organogenesis, or vacuum infiltration techniques that by-pass the need for tissue culture, are preferred.

Example 1. Lignin Modification of Alfalfa

Alfalfa plants were successfully transformed using the lignin-modifying transformation methods of the present invention. Alfalfa plants exhibiting changes in both

25 lignin content and composition were obtained.

To drive expression of transgenes in forage legumes, we chose the bean *PAL2* promoter, which was previously characterized as associated with lignification and strongly expressed in the vascular tissue of transgenic tobacco (Leyva, et al. 1992. "Cis-element combinations determine phenylalanine ammonia-lyase gene tissue specific expression

30 patterns," *Plant Cell* 4:263-271; Shufflebottom, et al. 1993. "Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell

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specificities and induction patterns," *Plant J* 3:835-845). A number of gene constructs were made, either to test the tissue specificity of the bean *PAL2* promoter in alfalfa using the reporter gene GUS, or to drive expression of the alfalfa O-methyltransferase genes COMT and/or CCOMT in the sense or antisense orientations. The bean *PAL2* promoter

5   was obtained from the genomic clone gPAL2 (Cramer, et al. 1989. "Phenylalanine ammonia-lyase gene organization and structure," *Plant Mol Biol* 12:367-383) and was cloned into the *EcoRI/BamHI* sites of pUC18. Site-directed mutagenesis was used to delete the *NdeI* site in pUC 18 to create the plasmid pUC 18-PAL. The GUS open reading frame was excised from the plasmid pGN100 (Reimann-Philipp, R. and Beachy, R.N.

10   1993. "Coat protein-mediated resistance in transgenic tobacco expressing the tobacco mosaic virus coat protein from tissue-specific promoters," *Mol Plant Microbe Interact* 6:323-330) by *EcoRI/SmaI* digestion, and two DNA polylinkers containing different restriction sites, *EcoRI-BgIll-NdeI-BamHI-SmaI* and *EcoRI-BgIll-BamHI-NdeI-SmaI*, were introduced independently between the *EcoRI* and *SmaI* sites, respectively. A

15   *BgIll/PstI* fragment containing the nopaline synthase *(nos)* terminator sequence was inserted into the *BamHI/PstI* sites of pUC18-PAL to give the plasmids pPTN1 and pPTN2, which contain the bean *PAL2* promoter and *nos* terminator. To create the cassette for *gus*A gene expression, the bean *PAL2* promoter was released from the plasmid pPTN2 by digestion with *EcoRI*, and the ends were filled in with Klenow fragment and then digested

20   with *BamHI*. The plasmid *ubi*3-GUS (Garbarino J.E. and Belknap W.R. 1994. "Isolation of a ubiquitin-ribosomal protein gene (ubi3) from potato and expression of its promoter in transgenic plants," *Plant Mol Biol* 24:119-127) was treated with *XbaI*, Klenow, and *BamHI* to replace the *ubi3* promoter with the isolated bean *PAL2* promoter. The *gus*A expression cassette was then cloned into *HindIII/EcoRI* cut pCAMB13 300 to create the *gus*A

25   expression construct pCAMGUS.

Constructs were introduced into *Agrobacterium tumefaciens* LBA4404 using the Gibco BRL Lifetechnologies electroporation procedure (Gibco BRL Lifetechnologies, Rockville, MD). Leaf disc transformation of alfalfa (cv Regen SY) was performed based on a method described previously (Shahin, et al. 1986 "Transformation of cultivated alfalfa

30   using disarmed *Agrobacterium tumefaciens,"* *Crop Sci* 26:1235-1239; Thomas, et al. 1990. "Selection of interspecific somatic hybrids of Medicago by using Agrobacterium-

transformed tissues," *Plant Sci* 69:189-198). Phosphinothricin (5mg/L) was added to the culture medium for selection of resistant transformants. Alfalfa plants were grown in the greenhouse under standard conditions. All transformations were performed with clonally propagated material of one selected highly regenerable line named 4D.

5   To confirm tissue specificity of the bean *PAL2* promoter in transgenic alfalfa, several independent plants were generated via *Agrobacterium*-mediated transformation with the pCAMGUS binary vector containing the GUS marker gene under control of the full length (-182 to -1226 bp) bean *PAL2* promoter, as illustrated in FIG. 2. Histochemical GUS assays were then performed to determine the cellular sites of *PAL2* promoter activity.

10   Hand sections of alfalfa stems, roots, and petioles were incubated on ice for 30 minutes in 2% paraformaldehyde and 100 mM Na-phosphate buffer, pH 7.0. They were then vacuum infiltrated in 2 mM X-gluc in 50 mM Na-phosphate buffer, pH 7.0, 0.5% Triton X-100 for 10 seconds, followed by a 2 hour incubation at 37°C. After staining, green tissues were bleached in 70% ethanol several times to allow visualization of the blue staining.

15   Transverse sections from these plants (containing the *PAL2-GUS* construct pCAMGUS) stained blue with the chromogenic substrate X-gluc revealed GUS expression in the vascular tissue of roots, stems, and petioles that was absent from similarly stained non-transgenic control tissue (containing empty pCAMBIA3300 vector). Although the majority of the staining in stem and petiole tissue was localized to vascular parenchyma

20   cells, there was also some staining of mesophyll cells and epidermal cells of petioles. These results were reproduced in other independent transformants. The relatively selective vascular tissue staining indicated that the bean *PAL2* promoter would be suitable for directing expression of COMT and CCOMT sense and antisense transgenes, as these enzymes are expressed in xylem and phloem parenchyma in alfalfa (Kersey, et al. 1999.

25   *Protoplasma* 209:46-57).

Full length alfalfa COMT and CCOMT cDNA sequences in the sense and antisense orientations were placed under control of the bean *PAL2* promoter in the binary vector pCAMBIA3300, as summarized in FIG. 2. Additional constructs contained tandem COMT and CCOMT cDNAs, in the sense or antisense orientations, with each cDNA

30   driven independently by a bean *PAL2* promoter, as shown in FIG. 2. The COMT and CCOMT coding sequences were isolated from separate OMT cDNA constructs in pET

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vectors (Inoue, et al. 1998. *Plant Physiol* 117:761-770), which contained the 1097 bp full length alfalfa COMT cDNA (Gowri, et al. 1991. *Plant Physiol* 97:7-14; GenBank Accession No. M63853) (SEQ ID NO:1 and FIG. 5) or the 743 bp full length alfalfa CCOMT cDNA (GenBank Accession No. U20736) (SEQ ID NO:2 and FIG. 6). The

5 COMT and CCOMT inserts were removed as *NdeI/BamHI* fragments and ligated into the *NdeI/BamHI* sites of pPTN1 and pPNT2, resulting in plasmids pPTNI-COMT and pPTNI-CCOMT for sense expression of COMT or CCOMT, respectively, and pPTN2-COMT and pPTN2-CCOMT for antisense expression of COMT or CCOMT, respectively. The chimeric genes were then cloned as *EcoRI/HindIII* fragments into the *EcoRI/HindIII* sites

10 of the binary vector pCAMBIA3300, which has a phosphinothricin resistance gene as selectable marker. Resulting binary constructs were designated pCAMC1 (single COMT, sense), pCAMC2 (single COMT, antisense), pCAMCCI (single CCOMT, sense), pCAMCC2 (single CCOMT, antisense), pCAMCICC1 (tandem COMT sense, CCOMT sense), pCAMC2CC2 (tandem COMT antisense, CCOMT antisense), and pCAMGUS, as

15 shown in FIG. 2.

To make constructs for sense or antisense expression of tandem COMT and CCOMT genes, plasmids pPTNI-COMT and pPTN2-COMT were first cut with *EcoRI*, filled in with the Klenow fragment of DNA polymerase I, and then digested with *HindIII*. The isolated fragments were ligated into *NarI*-treated, Klenow-treated, and *HindIII*-treated

20 pPTN1 to create the shuttle vector pPTN1-D. The tandem COMT and CCOMT region together with the *PAL2* promoter and *nos* terminator was cut out with *AatII*, filled in with Klenow, digested with *EcoRI* and finally ligated into *SmaI/EcoRI* cut pCAMBIA3300 to give binary expression constructs with both OMTs in the sense or antisense orientation. These were designated pCAMC1CC1 (tandem COMT sense, CCOMT sense) and

25 pCAMC2CC2 (tandem COMT antisense, CCOMT antisense). Introduction of both COMT and CCOMT transgenes into the same plant was also achieved by co-transformation using the above single COMT and CCOMT constructs. Constructs were introduced into alfalfa by *Agrobacterium*-mediated transformation of leaf discs followed by regeneration through somatic embryogenesis.

30 After regeneration (Thomas, et al. 1990. *Plant Science* 69:189-198) and transfer to the greenhouse, plants were first analyzed for integration of COMT and CCOMT

transgenes by polymerase chain reaction (PCR). The primers used were
5'-GGGTTCAACAGGTGAAACTC-3' and 5'-CCTTCTTAAGAAACTCCATGATG-3'
for COMT, and 5'-GGCAACCAACGAAGATCAAAAGC-3' and
5'-CTTGATCCTACGGCAGATAGTGATTCC-3' for CCOMT, which yielded diagnostic

5   1.1 kb or 0.75 kb amplification products in COMT or CCOMT transformants respectively.
Approximately 80% of the plants surviving selection were PCR-positive.

Internode samples (6th-9th internodes) from stems of putative transformants at the
same developmental stage were harvested and assayed for COMT and CCOMT enzymatic
activity, as shown in FIG. 3. Younger internodes (1st-4th) were excluded from the tissue

10   used for enzyme analysis, because these contain a second form of COMT that is not
recognized by the antiserum raised against the alfalfa COMT targeted by the present
transgenic strategy (Inoue, et al. 2000. *Arch Biochem Biophys* 375:175-182). Alfalfa stems
(internodes 6-9, counting from the first fully opened leaf at the top) were collected and
homogenized in liquid nitrogen. Powdered tissue was extracted for 1 hour at 4°C in

15   extraction buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2 mM DTT, 0.2 mM $MgCl_2$, 1
mM PMSF), and desalted on PD-10 columns (Pharmacia, Piscataway, NJ). Protein
concentrations were determined using Bradford dye-binding reagent (Bio-Rad) with
bovine serum albumin (BSA) as a standard. Enzyme activities were assayed essentially as
described elsewhere (Gowri, et al. 1991. *Plant Physiol* 97:7-14; Ni, et al. 1996. "Stress

20   responses in alfalfa *(Medicago sativa* L.) XXI. Activation of caffeic acid 3-O-
methyltransferase and caffeoyl CoA 3-O-methyltransferase genes does not contribute to
changes in metabolite accumulation in elicitor-treated cell suspension cultures," *Plant
Physiol* 112:117-726) with the following modifications. The assay mixtures contained 5 μl
of [$^{14}CH_3$]-S-adenosyl-L-Met (0.6 mM, 13 μCi/μmol), 5 μl of caffeic acid (1 mM) or

25   caffeoyl CoA (1 mM), 30 μl of assay buffer (100 mM Tris-HCl, pH 7.5, 10% glycerol, 2
mM DTT, 0.2 mM $MgCl_2$), and 5 μl protein extract. They were incubated at 30°C for 30
minutes, stopped by adding 50 μl of 0.2 M HCl (for COMT) or 10 μl of 3 M NaOH for
CCOMT, incubated at 37°C for 10 minutes, then (for CCOMT) acidified by adding 40 μl
of 1 M HCl. Labeled ferulic acid was extracted into 200 μl of hexane:ethyl acetate (1:1,

30   v/v), and 150 μl of the separated organic phases were transferred to scintillation vials for
determination of radioactivity.

There was a wide variation (nearly 4-fold) in COMT activity in a control population of 20 independent plants transformed with empty pCAMBIA3300 vector, as seen in FIG. 3A. Of twenty transformants containing the single COMT sense sequence shown in FIG. 3B, three lines (SC4, SC5, and SC52) had strongly reduced COMT

5   activities, whereas the remainder of the population exhibited, on average, a small increase in COMT activity compared to the average value for the control population. A similar situation was seen with respect to COMT activity in the double sense transformants shown in FIG. 3C, with one plant (DS14) showing strongly down-regulated COMT activity and the remainder of the population having a slightly elevated average COMT activity

10   compared to the controls. In the COMT antisense population shown in FIG. 3D, a single plant (AC310) had strongly reduced COMT activity, with the remainder of the overall population showing on average a small reduction when compared to the average value for the control population. In the double antisense lines (FIG. 3E), one plant (DA302) showed strongly reduced COMT activity.

15   There was less variation in CCOMT than in COMT activity in the control population, as seen by a comparison of FIG. 3A and FIG. 3F. Otherwise, the pattern of CCOMT activities in the transformants harboring sense and antisense CCOMT constructs was very similar to that observed for COMT. CCOMT activity was strongly down-regulated in two CCOMT sense lines (SCC 19 and SCC 20) as shown in FIG. 3G, in one

20   double sense line (DS 14) as shown in FIG. 3H, in two antisense lines (ACC305 and ACC315) as shown in FIG. 3I) and in one double COMT/CCOMT antisense line (DA302) as shown in FIG. 3J.

Transgene insertion was confirmed in selected COMT and CCOMT down-regulated alfalfa lines by Southern blot analysis. Total DNA was isolated from leaf tissue

'25   of each alfalfa line using a nucleon phytopure plant DNA extraction kit (Amersham; Arlington Heights, IL). DNA samples (7 μg) were digested with $EcoRI$, electrophoretically separated, and transferred to a nylon membrane (Hybond-N, Amersham) by standard procedures (Sambrook, et al. 1989. $Molecular\ Cloning.\ A\ Laboratory\ Manual$, 2nd Ed., New York, Cold Spring Harbor Laboratory Press). Blots

30   were probed with ^{32}P-labeled 1.1 kb alfalfa COMT or 0.75 kb CCOMT coding sequence probe and washed at high stringency conditions (final wash O.1 x SSC, 0.1% SDS, 65°C).

The probe was labeled with a ^{32}P-dCTP labeling kit (Amersham). A comparison of the results for selected control and COMT and/or CCOMT down-regulated transgenic lines showed transgene integration patterns indicative of multiple transgene insertions of COMT in independent transformants: SC4 (single COMT sense), SC5 (single COMT sense),

5   DS14 (double sense), DA302 (double antisense), and AC310 (single COMT antisense), all showing 3-5 unique bands. Similar transgene integration patterns were obtained showing multiple transgene insertions of CCOMT in independent transformants: DA302 (double antisense), ACC305 and ACC315 (single CCOMT antisense), and DS14 (double sense), all showing 1-5 unique bands.

10   RNA gel blot analysis confirmed that the reduced COMT or CCOMT activity in the various lines resulted from a severe reduction in COMT or CCOMT transcript levels. RNA was prepared from alfalfa leaves using TRIREAGENT (Molecular Research Center, Inc.) according to the manufacturer's suggested protocol. Total RNA samples (5-10µg) were fractionated on a formaldehyde denaturing gel according to standard protocols

15   (Sambrook, et al. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd Ed., New York, Cold Spring Harbor Laboratory Press), transferred to a Hybond-N nylon membrane, and hybridized with radiolabeled full length alfalfa COMT or CCOMT cDNA sequences at high stringency as for DNA gel blots. COMT transcripts were almost undetectable in the total RNA fraction from sense lines SC4, SC5, antisense line AC310, the double sense line

20   DS14 and the double antisense line DA302. CCOMT transcripts were likewise virtually undetectable in antisense lines ACC305 and ACC315, and in the double antisense line DA302. However, CCOMT transcripts were relatively unaffected in the double sense line DS14, in which CCOMT activity is reduced to approximately 23% of wild type.

Comparisons of COMT and CCOMT protein levels in the various transgenic lines

25   were carried out by western blot analysis. Crude proteins were extracted from the 6th to 9th internodes of selected individual transformants and two wild type plants, separated on 8-12% gradient SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were incubated in blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) for 2 hours, then incubated in blocking buffer with

30   monospecific polyclonal antisera raised against recombinant alfalfa COMT or CCOMT proteins for 2 hours (Kersey, et al. 1999. *Protoplasma* 209:46-57). The signals were

detected with ECL Western blotting detection reagents (Amersham) according to the manufacturer's protocol. The results indicated almost complete loss of COMT protein in the sense lines SC4, SC5 and SC52, in the antisense line AC310, in the double antisense line DA302, and in the double sense line DS14. CCOMT protein levels were almost

5   undetectable in the antisense lines ACC305 and ACC315, and were strongly reduced in the double antisense line DA302 and the double sense line DS14. Complete loss of CCOMT protein in the CCOMT antisense line ACC305 was unexpectedly accompanied by a strong increase in COMT protein level, and in COMT enzymatic activity (Table I). The above results indicate that expression of OMT sequences from the bean *PAL2* promoter results in

10   greater down-regulation of COMT and CCOMT than obtained in previous studies (Ni, et al. 1994.Transgenic *Res* 3:120-126; Atanassova, et al. 1995. *Plant J* 8:465-477;Van Doorsselaere, et al. 1995. *Plant J* 8:855-864; Zhong, et al. 1998. *Plant Cell* 10:2033-2045).

       Reduction of enzymatic activity resulting from reduced transcript levels in plants expressing gene constructs in the sense orientation is characteristic of epigenetic gene

15   silencing, which may occur at the transcriptional or post-transcriptional level (Vaucheret, et al. 1998. "Transgene-induced gene silencing in plants," *Plant J* 16:651-659). To determine the basis for the reduced COMT and CCOMT activities in some of the sense transgenic lines, nuclear run-on transcription analyses were performed with transcripts completed in vitro from nuclei isolated from wild type and COMT-suppressed or CCOMT-

20   suppressed sense lines SC4 and SCC19. Nuclei were isolated from fresh leaf tissue as described by Cox and Goldberg (Cox, K.H. and Goldberg, R.B. 1988. "Analysis of plant gene expression," *Plant Molecular Biology. A Practical Approach.* C.H. Shaw, ed, Oxford, IRL Press, pp. 1-35). Run-on transcription reaction mixtures contained 125 µl nuclei, 30 µl of 1 M $(NH_4)_2SO_4$, 12 µl of 100 mM $MgCl_2$, 3 µl of 100 µM phosphocreatine, 12 µl of

25   creatine phosphate kinase (0.25 mg/ml), 15 µl of RNasin (Promega; Madison, WI), 30 µl of 5 mM CTP, GTP and ATP mixture, 48 µl of water and 25 µl of ^{32}P-UTP (NEN, 10 µCi/µl). The reaction mixture was incubated at 30°C for 30 minutes, then treated with RNase-free DNase (30 units, Promega) and proteinase K (500 µg, GibcoBRL) at 30°C for 20 minutes. RNA transcripts were extracted with an equal volume of phenol-chloroform

30   (1:1), and extracted again with an equal volume of chloroform. Unincorporated nucleotide was removed by filtration through Sephadex G-50 (Amersham). Radioactivity

incorporated into the synthesized RNA was then measured by slot blot hybridization. Two hundred ng of COMT, CCOMT or β-ATPase (positive control) cDNAs were denatured and transferred to a nitrocellulose membrane by UV cross-linking, and hybridized with radiolabeled RNA probe. Hybridization and washes were carried out at 65°C according to

5   Church and Gilbert (Church, G.H. and Gilbert, W. 1984. "Genomic sequencing," *Proc Natl Acad Sci USA* 81:65-71). Autoradiographs were quantified using a Molecular Dynamics phosphorimager. The results indicated that the transcription rates of both COMT and CCOMT were essentially the same in wild type and down-regulated lines. However, the data from the RNA gel blot analysis mentioned above indicated that the steady state

10   transcript levels in the sense COMT and CCOMT lines were only a fraction of the control levels, consistent with post-transcriptional gene silencing being responsible for reduced COMT and CCOMT expression in the sense transgene lines.

Table I summarizes the COMT and CCOMT activity, lignin content, and lignin composition of selected transgenic alfalfa lines harboring alfalfa COMT and CCOMT

15   sequences in the sense or antisense orientations. Levels of acetyl bromine (AcBr) lignin and Klason lignin are expressed as % of dry matter. Levels of S, G and 5OHG are expressed as mmol/g dry weight. Down-regulation of COMT had no effect on the activity of CCOMT, and vice-versa, with one notable exception. The reduction of CCOMT to less than 4% of wild type activity in line ACC305 was associated with an approximate

20   doubling of COMT activity as compared to wild-type levels, a finding consistent with the western blot data noted above.

Lignin content in the various lines was determined according to standard procedures for Klason and acetyl bromide soluble lignin (Lin, S.Y. and Dence, C.W. eds, *Methods in Lignin Chemistry,* Springer Series in Wood Science, Springer-Verlag, Berlin,

25   Heidelberg, 1992). Two hundred milligrams of dried sample was used for lignin analysis, and Klason lignin content was calculated as weight percentage of the extract-free sample.

Klason lignin levels of three independent control lines averaged 17.6% of dry matter; this value was reduced to between 15.3% and 12.5% in all lines with down-regulated COMT or CCOMT activity. The largest reductions in Klason lignin (down to

30   70% of the wild type value) were in lines with gene silenced COMT. However, Klason lignin was also reduced in line ACC305, which has only 3.6% of the wild type CCOMT

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Table I: COMT and CCOMT Activities of Select Independent Transgenic Alfalfa Lines

	COMT pkat/mg	CCOMT pkat/mg	AcBr Lignin%	Klason Lignin%	S Lignin	G Lignin	5-OH Lignin	S/G
1	5.98	22.35	16.07	17.21	158.8	305.4	0	0.52
2	6.55	23.77	17.79	17.91	152.8	325.2	0	0.47
48	8.19	21.13	17.52	17.64	156.2	279.4	0	0.56
SC4	1.06	20.11	16.48	12.67	10.4	227.9	7.4	0.05
SC5	1.24	22.26	16.35	12.46	8.6	246.1	8.8	0.04
SC52	1.19	22.36	16.83	14.15	17.1	223.7	1.8	0.07
AC310	0.31	22.17	16.97	15.30	0	248.2	0	0
ACC305	14.39	0.78	16.36	14.58	159.1	150.2	0	1.05
ACC315	8.06	10.7	15.31	15.50	164.0	243.6	0	0.69
DS14	0.78	5.59	16.54	14.72	54.0	223.7	0	0.23
DA302	0.81	1.15	16.78	15.06	14.0	303.0	0	0.05

activity but nearly double the wild type COMT activity, and in line AC315, with less than 5% wild type CCOMT activity but normal COMT activity. Thus, reductions in either COMT or CCOMT activities can independently reduce Klason lignin levels in alfalfa. In contrast to the effects on Klason lignin, down-regulation of neither OMT appeared to have
5   a significant effect on acetyl bromide extractable lignin.

A qualitative and semi-quantitative analysis of the lignin in the transgenic alfalfa lines was made using histochemical staining methods. Histochemical analysis of lignin in transverse stem sections (5th internode) of control (wild type), antisense COMT line AC310, and antisense CCOMT line ACC305 alfalfa was performed as follows. For Maule
10   reagent staining, hand sections of alfalfa stems were immersed in 1% (w/v) potassium permanganate solution for 5 minutes at room temperature, then washed twice with 3% hydrochloric acid until the color turned from black or dark brown to light brown. Phloroglucinol-HCl reagent was prepared by mixing two volumes of 2% (w/v) phloroglucinol in 95% ethanol with one volume of concentrated HCl. Photographs were
15   taken within 30 minutes of staining. Staining of transverse stem sections with phloroglucinol-HCl indicated little or no reduction in staining intensity in COMT or CCOMT antisense as compared to control lines. Reduction in phloroglucinol staining is often taken as being indicative of a reduction in lignin content, although the reagent appears most specific for coniferaldehyde end groups in lignin (Lewis, N.G. and
20   Yamamoto, E. 1990. "Lignin: occurrence, biogenesis and biodegradation," *Annu Rev Plant Physiol Plant Mol Biol* 41:455-496). In contrast, staining with Maule reagent gave a red coloration in wild type plants which was lost in COMT down-regulated lines. Such a color shift is reported to be diagnostic for reduction of S lignin (Lewis, N.G. and Yamamoto, E. 1990. *Plant Physiol Plant Mol Biol* 41:455-496).
25   Analysis of lignin degradation products by gas chromatography/mass spectrometry (GC/MS) following thioacidolysis is a widely used method for analysis of lignin monomer composition (Lapierre, et al. 1985. "Thioacidolysis of lignin: Comparison with acidolysis," *J Wood Chem Technol* 5:277-292), and can be extended to analyze dimer linkage patterns. Thioacidolysis and the Raney nickel desulfurization method of Lapierre et al. (Lapierre, et
30   al. 1995. "New insight into the molecular architecture of hardwood lignins by chemical degradative method," *Res Chem Intermed* 21:397-412) were therefore used to determine

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lignin composition and resistant inter-unit bonds in the selected transgenic alfalfa lines. The data from such analyses shown in FIG. 4 and Table I indicate that reduction in lignin levels in plants with down-regulated COMT activity is associated with a much greater decrease in S units than in G units, resulting in a large decrease in S/G ratio, consistent

5   with the results of histochemical staining with Maule reagent. In fact, thioacidolysis products of S lignin were not detected at all in the COMT antisense line AC310. In contrast, there was no reduction in S lignin in lines with reduced CCOMT activity, unless there was a corresponding decrease in COMT activity, as in the double sense and antisense lines. However, levels of G lignin were most strongly reduced in transgenic line ACC305,

10   the line with the greatest decrease in CCOMT activity. Overall, the data clearly indicate that COMT down-regulation impacts both S and G lignin, with greatest effects on S lignin, whereas CCOMT down-regulation only affects G lignin in alfalfa. Reduction of CCOMT to less than 5% of wild-type activity leads to reductions in G lignin with no apparent effect on S lignin in alfalfa. This contrasts with reported reductions in both G and S lignin in

15   transgenic tobacco down-regulated in CCOMT expression (Zhong, et al. 1998. *Plant Cell* 10:2033-2045). CCOMT would, therefore, appear to function in the biosynthesis of G lignin in alfalfa, as has been previously proposed in tobacco (Ye, et al. 1994. *Plant Cell* 6:1427-1439; Zhong, et al. 1998. *Plant Cell* 10:2033-2045) but not in S lignin biosynthesis, contrary to the model of Li, et al. based on in vitro studies of enzyme

20   specificity (Li, et al. 2000. *J Biol Chem* 275:6537-6545).

Analysis of gas chromatogram traces from the thioacidolysis reactions revealed new peaks in the reaction products from lignin extracted from COMT downregulated plants, as shown in FIG. 4. These peaks were identified as originating from 5-hydroxyguaiacyl moieties that might be expected to be present if S lignin biosynthesis

25   were being blocked primarily at the second methylation stage in COMT down-regulated plants. However, the levels of 5-hydroxyguaiacyl units were always much less than the corresponding reduction in S units, as shown in Table I.

In the intact lignin polymer, the various monomeric units are linked to each other through covalent bonding at a number of different positions. This gives rise to more than five

30   major types of lignin dimers that can be analyzed by GC/MS after thioacidolysis and Raney nickel desulfurization, as illustrated by the five structures in Table II. 5-5 and 4-*O*

5 linkages only occur between G units, whereas β-β linkages only occur between S units. β-1, β-5 and β-6 linkages can occur between two G units or between a G and an S unit. Thus, the five basic linkage types can result in nine different lignin dimers. The levels of these various dimers were analyzed by GC/MS, from the series of control and COMT or

5   CCOMT down-regulated alfalfa plants previously analyzed for lignin content and monomer composition. Table II depicts the dimer bonding patterns of lignin samples from wild type, COMT-suppressed, and CCOMT-suppressed alfalfa plants following determination of dimer composition by thioacidolysis followed by Raney nickel desufurization. Units are mmol/g dry weight. The Klason lignin levels and S/G ratios of

10   the various lines are given in Table I. The chemical structures of a selection of the dimer linkages recovered from lignin after thioacidolysis and Raney nickel desulfurization are shown. The results in Table II indicate that reduction of COMT activity resulted in at most a small increase in the recovery of dimers consisting of two G units (5-5, 4-O-5, β-1 (G), β-5 (G), β-6 (G)). However, there was a total loss of recovered dimers with β-β or mixed

15   β-1 or β-6 linkages, which all involve S units, in plants with reduced COMT activity. In contrast, reduction of CCOMT activity did not lead to a reduction in dimers containing S units. Rather, CCOMT down-regulation appeared to lead to increased recovery of β-5 (G) dimers but a reduction in β-6 (G) dimers. Lignin from line ACC305 had the highest proportion of β-β linked S units.

20   Taken together, the above data indicate that the reduction in S/G ratio caused by down-regulation of COMT results in a decreased proportion of linkages involving S units. This indicates that lignin linkage pattern is determined by monomer availability. However, there were also qualitative changes in lignin dimers resulting from OMT down-regulation. Thus, gas chromatograms of thioacidolysis/Raney nickel desulfurization products of lignin

25   from five independent COMT down-regulated plants exhibited a major peak at 52.9 minutes retention time that was absent from corresponding traces from wild type or CCOMT down-regulated plants. The compound was analyzed by MS and shown to have a molecular ion with a mass/charge ratio (m/z) of 504, identical to that of the γ-p-coumarate ester of an S unit, a dimer previously identified in maize lignin (Grabber, et al. 1996. "p-

30   Coumaroylated syringyl units in maize lignin: implications for β-ether cleavage by thioacidolysis," *Phytochemistry* 43:1189-1194). However, the retention time of the new

Table II: Dimer Bonding Patterns of Lignin Samples from Wild Type, COMT-suppressed and CCOMT-suppressed Alfalfa

Line	5-5,G	4-0-5,G	b-1,G	b-1,M	b-5,G	b-5,M	b-b,S	b-6,G	b-6,S	H-S,ester
2	13	3.6	17.9	3.2	19.4	16.9	9.6	11	5	0
48	13.3	3.6	17.5	2.4	20.4	14	9.5	12.4	6.3	0
SC4	16.6	4.1	21.9	0	24.6	19	0	14.2	0	9.4
SC5	17.3	3.6	20.3	0	24.7	14	0	19.2	0	7.1
AC310	15.5	3.4	19.1	0	23.7	15	0	10.6	0	12.4
DA302	16.9	4	21.9	0	30.7	6.4	0	9	0	10.5
DS14	15	3.3	20.7	0	21.5	18.9	0	12.8	0	7.5
ACC305	11.2	2.9	18.6	5.2	22.2	19.5	14.8	10.1	6.6	0
ACC315	14.8	3.6	23.5	5.7	30.1	10.3	4.5	6.2	3.1	0
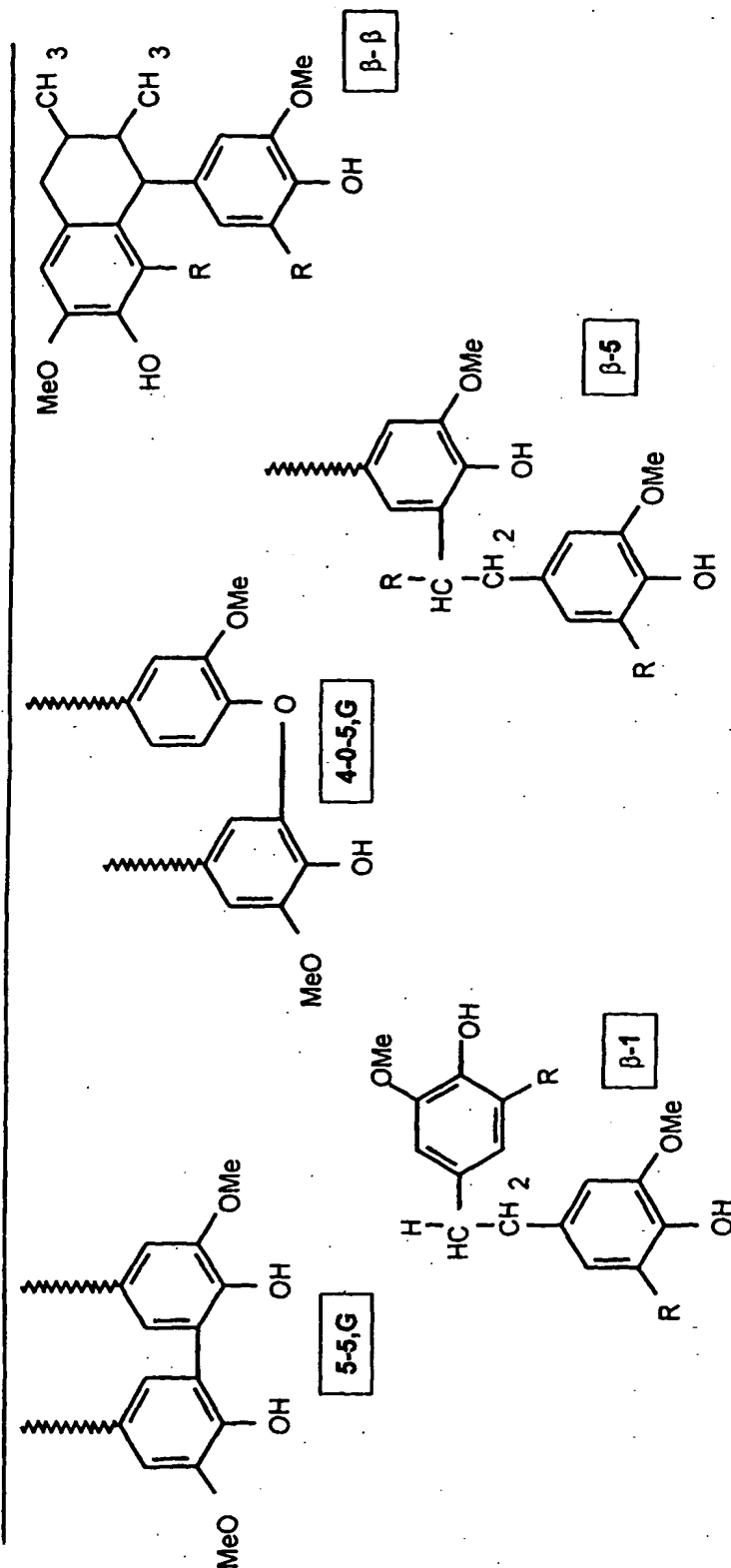
dimer and its MS fragmentation pattern were similar but not identical to those of an authentic sample of the S unit coumarate ester. The appearance of the new dimer correlated with the loss of S-linked dimers from the lignin in COMT down-regulated plants (Table II).

5   On the basis of the above analyses, line SC5 was chosen as a severely COMT down-regulated line in which recoverable S lignin was virtually absent, and line ACC305 chosen as a severely CCOMT down-regulated line in which G lignin was reduced and S/G ratio increased. Line CK48 was chosen as a control. The lines were vegetatively propagated, and greenhouse grown plants were harvested at the late bud stage, dried at

10   120°F, ground into 1 mm powder and put into preweighed nylon bags (approximately 5g/bag). These bags were put into the rumens of fistulated steers for 12, 24, 36, or 72 hours of digestion. At each time point, duplicate samples for each line were analyzed in three different steers. After digestion, bags were taken out from the rumen, washed in a commercial washing machine and vacuum-dried in a freeze drier. Digestibility was

15   calculated based on sample weight before and after digestion. The results in Table III show that total digestion of forage from all three lines reached a value of approximately 80% by 12 hours within the rumen. However, there was no further digestion of forage from the control and COMT down-regulated lines beyond 24 hours in the rumen. In contrast, the forage from the CCOMT down-regulated line continued to be digested up to

20   at least 76 hours within the rumen, attaining a value of approximately 89% digestibility.

Down-regulation of CCOMT by antisense or gene-silencing approaches was shown to be a valid method for improving forage digestibility in alfalfa, and presumably other forage legumes such as clovers and trefoils. The lack of effectiveness of strong down-regulation of COMT in significantly improving forage digestibility indicates that, contrary

25   to current opinion, reducing S lignin is not a valid strategy for improving digestibility. Rather, it is the reduction in G lignin, which may result in a reduced level of lignin condensation, that has the major impact on digestibility of alfalfa.

Table III: *In vivo* Digestibility of Alfalfa in Fistulated Steers

Plant Line	Time in Rumen	Steer1	Digesitibility Steer2	(%) Steer3	Average
CK48	12h	80.00	78.19	80.83	79.68
	24h	83.04	82.95	83.87	83.29
	36h	83.98	83.20	83.91	83.70
	72h	84.15	82.34	82.02	82.83
SC5	12h	78.89	77.21	81.52	79.21
	24h	84.39	84.82	85.75	84.99
	36h	84.27	84.92	85.00	84.73
	72h	85.12	85.00	84.35	84.82
ACC305	12h	80.91	77.97	81.91	80.26
	24h	84.26	85.21	87.21	85.56
	36h	86.22	86.86	87.21	86.76
	72h	88.55	90.92	87.74	89.07

WE CLAIM:

1. A method for modulating the lignin content of a forage legume comprising
transforming a forage legume cell with a vector comprising a lignification-
associated tissue specific promoter functionally linked to a DNA construct comprising at
least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme
5 or a fragment thereof; and
generating plants from said transformed forage legume cell

2. The method of Claim 1, wherein said forage legume cell is co-transformed with
a vector comprising a lignification-associated tissue specific promoter functionally linked
to a DNA construct comprising at least one open reading frame encoding for a *Medicago
sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof.

3. A method for modulating the lignin content of a forage legume comprising
transforming a forage legume cell with a vector comprising a lignification-
associated tissue specific promoter functionally linked to a DNA construct comprising at
least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-
5 methyltransferase enzyme or a fragment thereof; and
generating plants from said transformed forage legume cell.

4. A method for modulating the lignin content of a forage legume comprising
transforming a forage legume cell with a vector comprising a DNA construct
comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-
methyltransferase enzyme or a fragment thereof under expression control of a first
5 lignification-associated tissue specific promoter and at least one open reading frame
encoding for a caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under
expression control of a second lignification-associated tissue specific promoter, wherein
said first and second lignification-associated tissue specific promoter can be the same or
different; and
10 generating plants from said transformed forage legume cell.

31

5. A method for producing a forage legume having altered lignin composition comprising

transforming a forage legume cell with a DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or a

5   fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

6. The method of Claim 5, wherein said forage legume cell is co-transformed with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression control of a lignification-associated tissue specific promoter.

7. A method for producing a forage legume having altered lignin composition comprising

transforming a forage legume cell with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase

5   enzyme or a fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

8. A method for producing a forage legume having altered lignin composition comprising

transforming a forage legume cell with a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme

5   or a fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression control of a second lignification-associated tissue specific promoter to form a transgenic cell, wherein said first and second lignification-associated tissue specific promoter can be the same or different;

10   and

32

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

9.   A method for improving the digestibility of forage legumes comprising stably incorporating into the genome of said forage legume a DNA construct comprising at least one open reading frame encoding for a 3-O-methyltransferase enzyme or a fragment thereof from the lignin biosynthetic pathway under expression control of a lignification-
5   associated tissue specific promoter, wherein expression of said enzyme or fragment produces a change in lignin composition in said forage legume.

10. The method of Claim 9, wherein said enzyme is caffeoyl CoA 3-O-methyltransferase.

11. The method of Claim 10, wherein said forage legume is co-transformed with at least one open reading frame encoding for a caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter.

12. The method of Claim 9, wherein said DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or a fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a caffeic acid 3-O-
5   methyltransferase enzyme or a fragment thereof under expression control of a second lignification-associated tissue specific promoter, wherein said first and second lignification-associated tissue specific promoter can be the same or different.

13. The method of Claim 9, 10, 11 or 12, wherein guaiacyl lignin content is reduced.

14. A method for producing a woody plant having altered lignin composition comprising
       transforming a woody plant cell with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeoyl CoA 3-O-methyltransferase
5   enzyme or fragment thereof under expression control of a lignification-associated tissue

33

specific promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

15. The method of Claim 14, wherein said woody plant cell is co-transformed with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression control of a lignification-associated tissue specific promoter.

16. A method for producing a woody plant having altered lignin composition comprising

transforming a woody plant cell with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme

5   or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

17. A method for producing a woody plant having altered lignin composition comprising

transforming a woody plant cell with a DNA construct comprising in tandem at least one open reading frame encoding for a *Medicago sativa* caffeoyl CoA 3-O-

5   methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter and at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated tissue specific promoter to form a transgenic cell, wherein said first and second lignification-associated

10   tissue specific promoter can be the same or different; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

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18. A method for modulating the lignin content of a woody plant comprising

transforming a woody plant cell with a DNA construct comprising at least one open

reading frame encoding for a *Medicago sativa* caffeoyl CoA 3-O-methyltransferase

enzyme or fragment thereof under expression control of a lignification-associated tissue

5   specific promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and

plant growth.

19. The method of Claim 18, wherein said woody plant cell is co-transformed with

a DNA construct comprising at least one open reading frame encoding for a *Medicago*

sativa caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression

control of a lignification-associated tissue specific promoter.

20. A method for modulating the lignin content of a woody plant comprising

transforming a woody plant cell with a DNA construct comprising at least one open

reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme

or fragment thereof under expression control of a lignification-associated tissue specific

5   promoter to form a transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and

plant growth.

21. A method for modulating the lignin content of a woody plant comprising

transforming a woody plant cell with a DNA construct comprising in tandem at

least one open reading frame encoding for a *Medicago sativa* caffeoyl CoA 3-O-

methyltransferase enzyme or fragment thereof under expression control of a first

5   lignification-associated tissue specific promoter or fragment thereof and at least one open

reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme

or fragment thereof under expression control of a second lignification-associated tissue

specific promoter to form a transgenic cell, wherein said first and second lignification-

associated tissue specific promoter can be the same or different; and

10   cultivating said transgenic cell under conditions conducive to regeneration and

plant growth.

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22. A method for making lignins with altered dimer bonding patterns comprising transforming a plant cell with a DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a

5   transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

23. The method of Claim 22, wherein said plant cell is co-transformed with a DNA construct comprising at least one open reading frame encoding for a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof under expression control of a lignification-associated tissue specific promoter.

24. A method for making lignins with altered dimer bonding patterns comprising transforming a plant cell with a DNA construct comprising at least one open reading frame encoding for a caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a lignification-associated tissue specific promoter to form a

5   transgenic cell; and

cultivating said transgenic cell under conditions conducive to regeneration and plant growth.

25. A method for making lignins with altered dimer bonding patterns comprising transforming a plant cell with a DNA construct comprising in tandem at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or fragment thereof under expression control of a first lignification-associated tissue specific promoter

5   and at least one open reading frame encoding for a caffeic acid 3-O-methyltransferase enzyme or fragment thereof under expression control of a second lignification-associated tissue specific promoterto form a transgenic cell, wherein said first and second lignification-associated tissue specific promoter can be the same or different; and

cultivating said transgenic cell under conditions conducive to regeneration and

10   plant growth.

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26. The method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25, wherein the open reading frame is in a sense orientation.

27. The method of Claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25, wherein the open reading frame is in an antisense orientation.

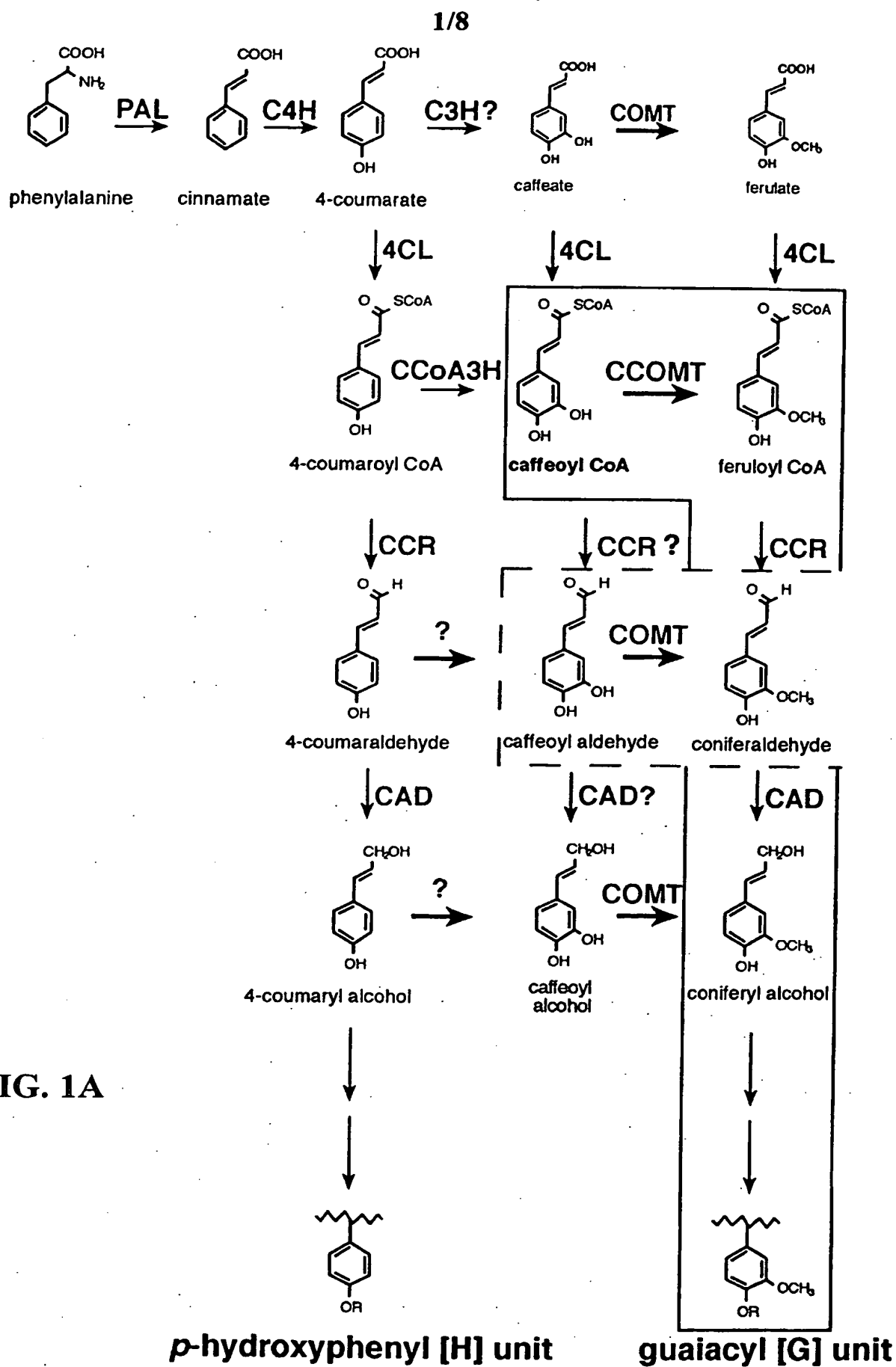28. The method of Claim 26, wherein said promoter is a bean *PAL2* promoter.

29. The method of Claim 27, wherein said promoter is a bean *PAL2* promoter.

30. A plant transformed by the method of Claim 26.

31. A plant transformed by the method of Claim 27.

32. A plant transformed by the method of Claim 28.

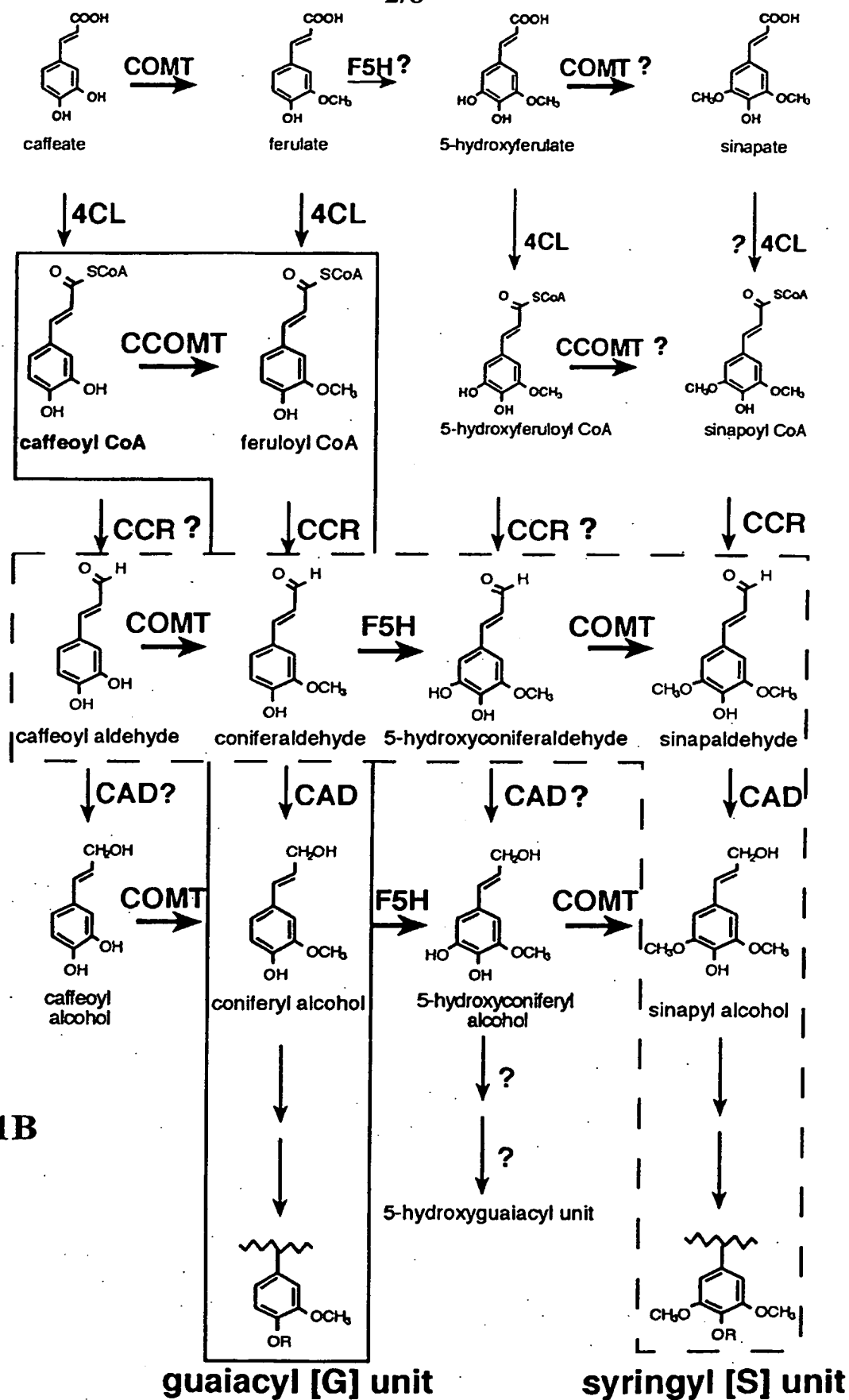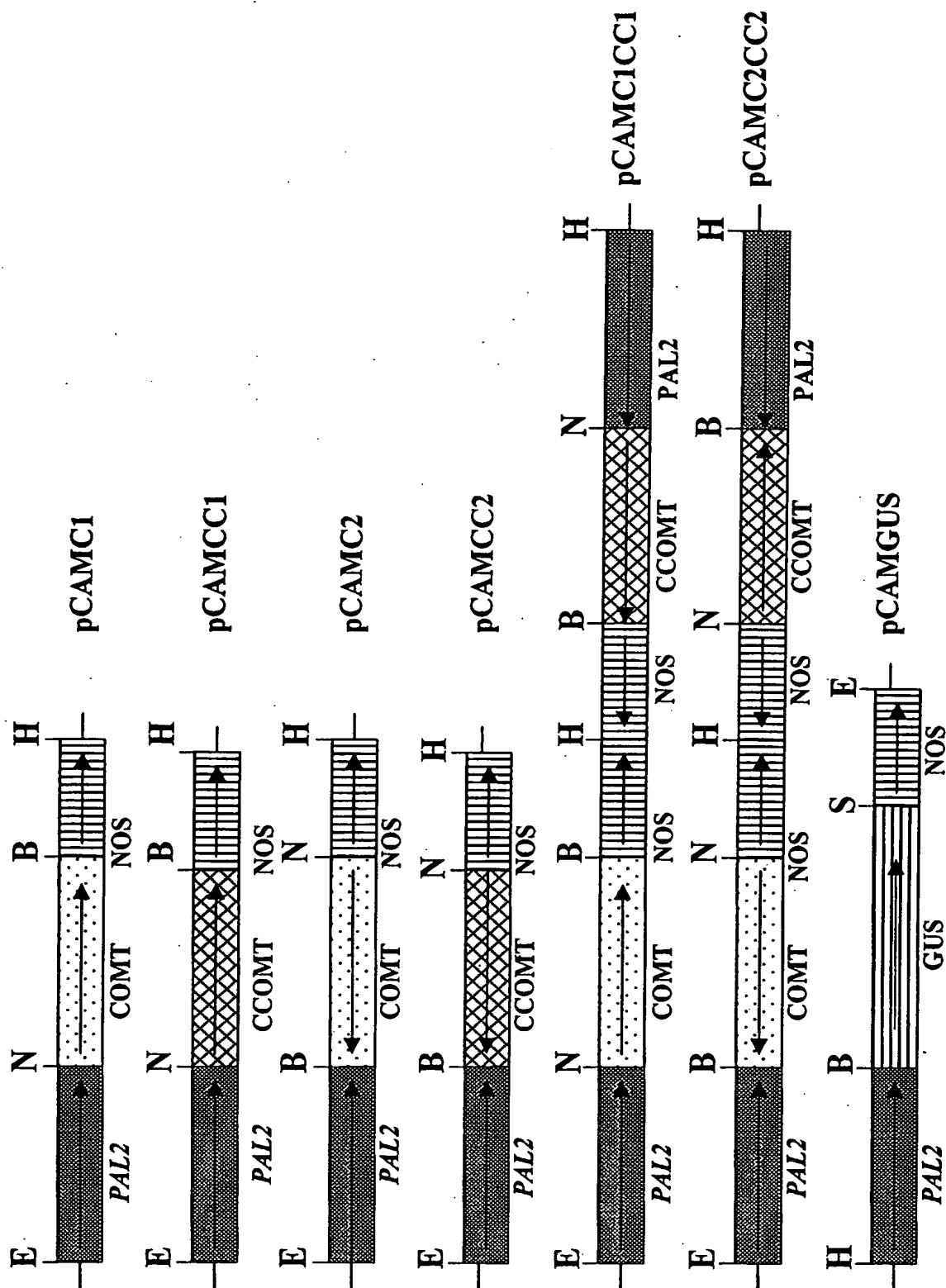33. A plant transformed by the method of Claim 29.

1/8

FIG. 1A

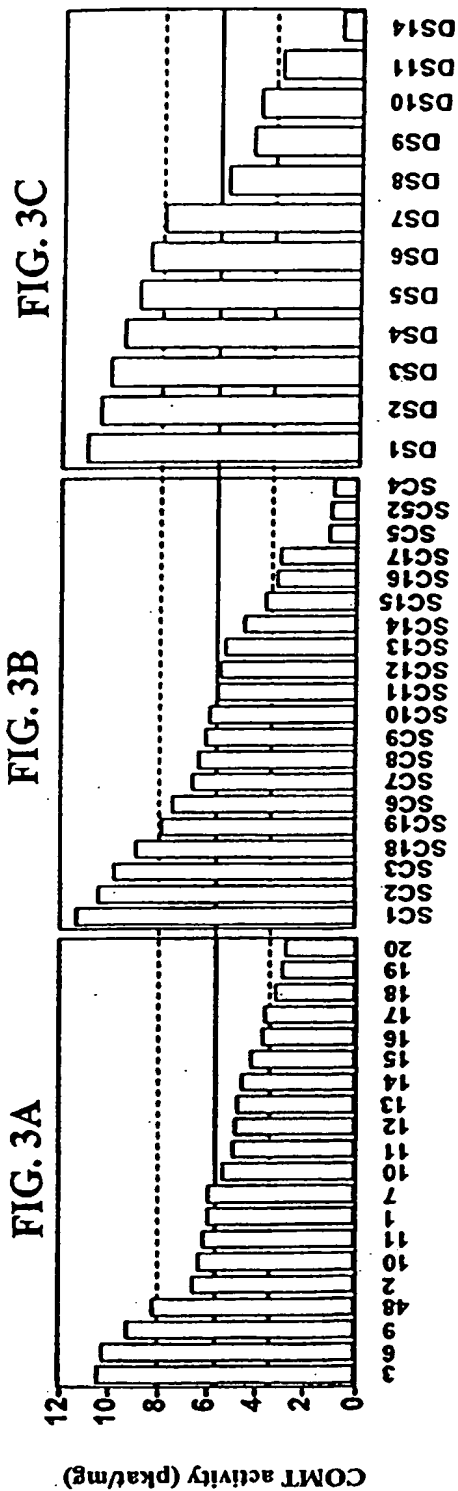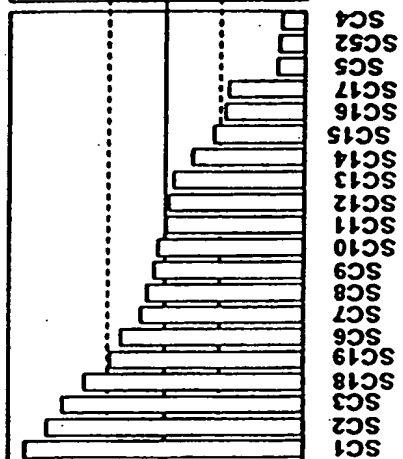*p*-hydroxyphenyl [H] unit   guaiacyl [G] unit

● ●

FIG. 1B

guaiacyl [G] unit   syringyl [S] unit

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FIG. 2

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

Plant number

COMT activity (pkat/mg)

COMT activity (pkat/mg)

FIG. 3H
FIG. 3G
FIG. 3F

FIG. 3J
FIG. 3I

Plant number

CCOMT activity (pkat/mg)
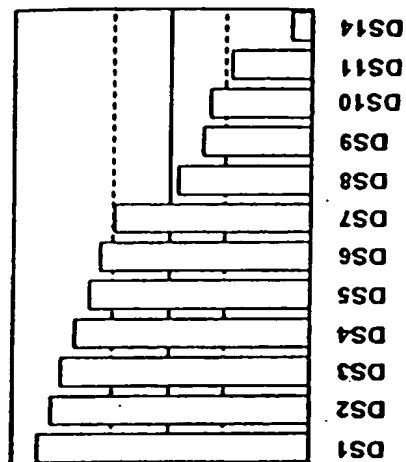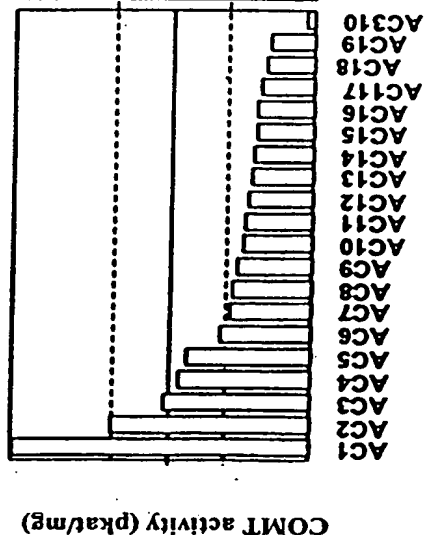
FIG. 4

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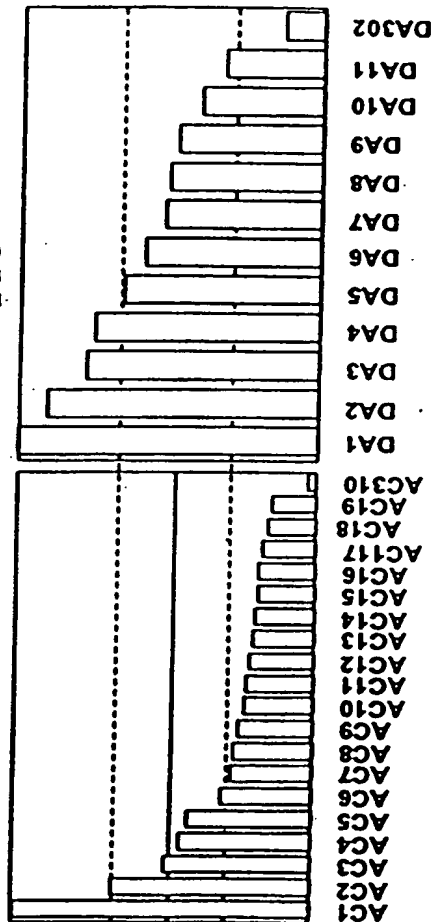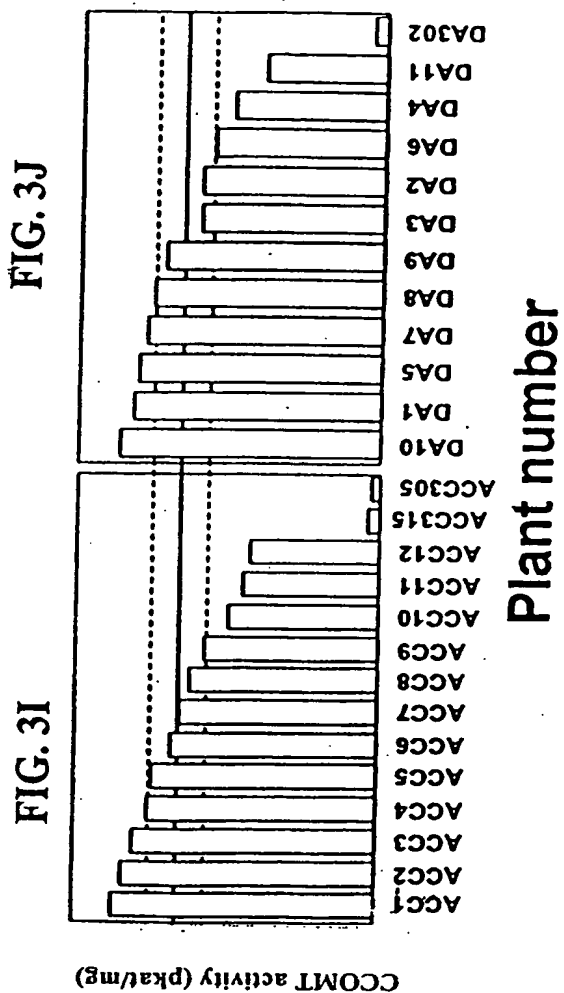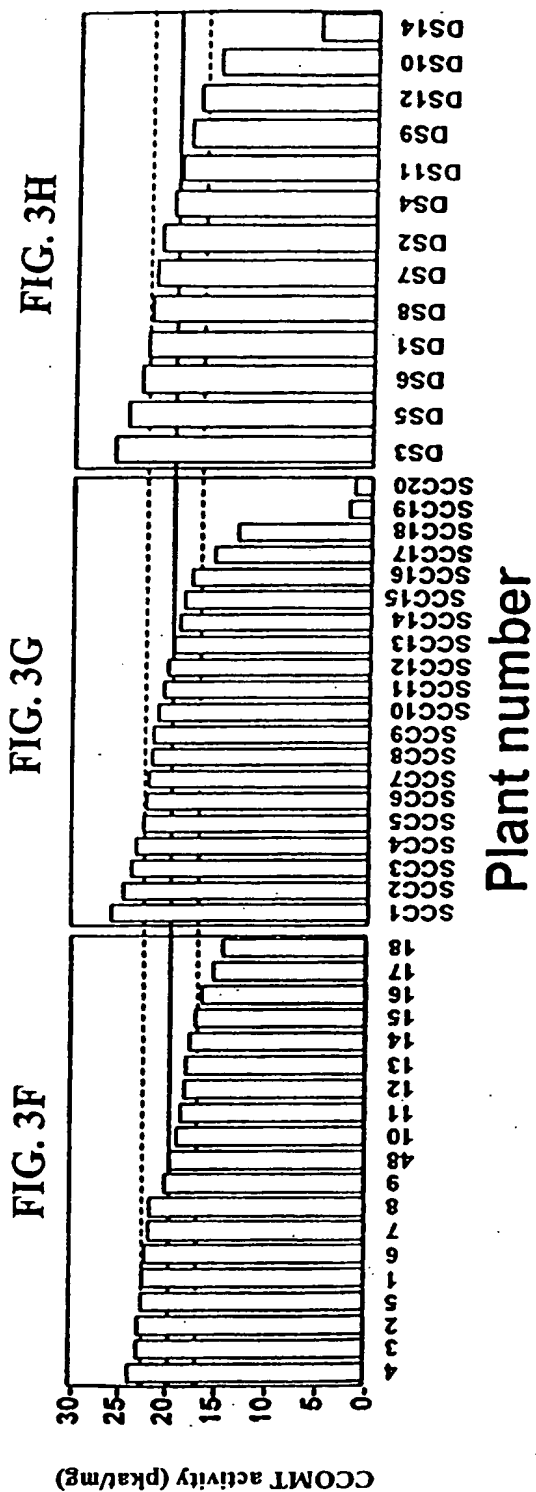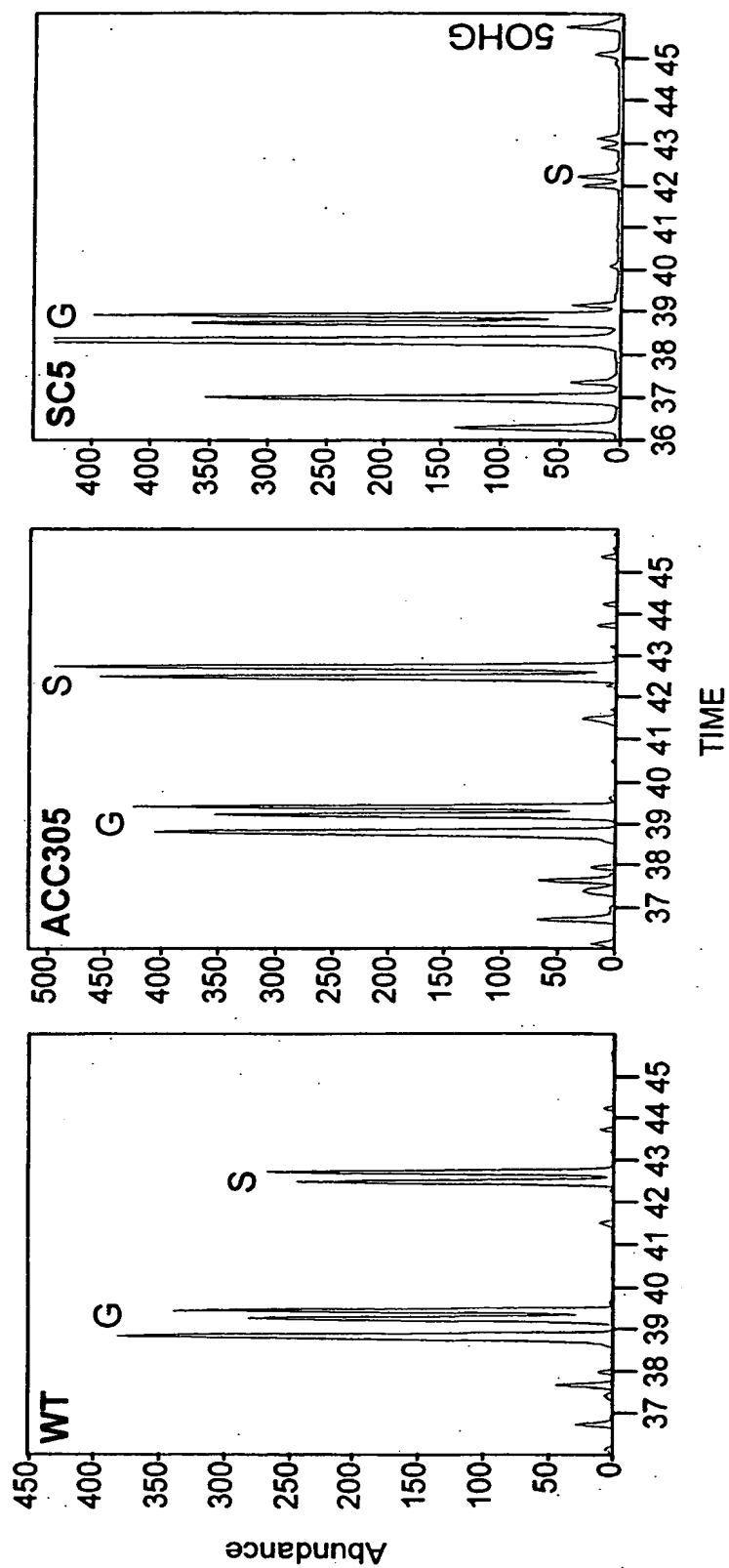
FIG. 5

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FIG. 6

SEQUENCE LISTING

<110> The Samuel Roberts Noble Foundation, Inc.
      Dixon, Richard A.
      Guo, Dianjing

<120> Method for Modifying Lignin Composition and Increasing In Vivo
Digestibility of Forages

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<150> US 60/192,086
<151> 2000-03-24

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NSDOCID -WO 017309043 1

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Val Asp His Lys Ile Asp Phe Arg Glu Gly Pro Ala Leu Pro Val Leu
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Asp Glu Met Ile Lys Asp Glu Lys Asn His Gly Ser Tyr Asp Phe Ile
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Phe Val Asp Ala Asp Lys Asp Asn Tyr Leu Asn Tyr His Lys Arg Leu
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caa caa gat gga aag gtt cag aga ctt tat ggt ttg gct act gtt gct     336
Gln Gln Asp Gly Lys Val Gln Arg Leu Tyr Gly Leu Ala Thr Val Ala
                100                 105                 110

aag tat ttg gtt aag aat gaa gat ggt gta tcc att tct gct ctt aat     384
Lys Tyr Leu Val Lys Asn Glu Asp Gly Val Ser Ile Ser Ala Leu Asn
            115                 120                 125

ctc atg aat cag gat aaa gtg ctc atg gaa agc tgg tac cac cta aaa     432
Leu Met Asn Gln Asp Lys Val Leu Met Glu Ser Trp Tyr His Leu Lys
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gat gca gtc ctt gat ggg ggc att cca ttc aac aag gct tat gga atg     480
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Asn Lys Gly Met Ser Asp His Ser Thr Ile Thr Met Lys Lys Ile Leu
            180                 185                 190

gag acc tac aca ggt ttt gaa ggc ctt aaa tct ctt gtt gat gta ggt        624
Glu Thr Tyr Thr Gly Phe Glu Gly Leu Lys Ser Leu Val Asp Val Gly
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ggt ggt act gga gct gta att aac acg att gtc tca aaa tat ccc act        672
Gly Gly Thr Gly Ala Val Ile Asn Thr Ile Val Ser Lys Tyr Pro Thr
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ata aag ggt ata aat ttt gat tta ccc cat gtc att gaa gat gct cca        720
Ile Lys Gly Ile Asn Phe Asp Leu Pro His Val Ile Glu Asp Ala Pro
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Pro Met Ile Leu Lys Ser Ala Leu Glu Leu Asp Leu Leu Glu Ile Ile
        35                  40                  45


Ala Lys Ala Gly Pro Gly Ala Gln Ile Ser Pro Ile Glu Ile Ala Ser
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Gln Leu Pro Thr Thr Asn Pro Asp Ala Pro Val Met Leu Asp Arg Met
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Leu Arg Leu Leu Ala Cys Tyr Ile Ile Leu Thr Cys Ser Val Arg Thr
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Gln Gln Asp Gly Lys Val Gln Arg Leu Tyr Gly Leu Ala Thr Val Ala
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Ser Tyr Pro Gly Val Glu His Val Gly Gly Asp Met Phe Val Ser Ile
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Pro Lys Ala Asp Ala Val Phe Met Lys Trp Ile Cys His Asp Trp Ser
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Asp Glu His Cys Leu Lys Phe Leu Lys Asn Cys Tyr Glu Ala Leu Pro
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

[Continued on next page]

(54) Title: METHOD FOR MODIFYING LIGNIN COMPOSITION AND INCREASING IN VIVO DIGESTIBILITY OF FORAGES

(57) Abstract: Methods for transforming forage legumes or woody plants with a DNA construct comprising at least one open reading frame encoding for a caffeoyl CoA 3-O-methyltransferase enzyme or a *Medicago sativa* caffeic acid 3-O-methyltransferase enzyme or a fragment thereof in either a sense or antisense orientation under a lignification-associated tissue specific promoter have been found, resulting in the down-regulation of the corresponding homologous gene either through antisense inhibition or sense suppression, as well as reduced lignin content and modified lignin composition in the transgenic plants. The expression of the caffeoyl CoA 3-O-methyltransferase transgene produces an increased syringyl lignin to guaiacyl lignin ratio in the transformed plant, and greatly improved forage digestibility.

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   C12N15/82   A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   C12N   A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 03535 A (PURDUE RESEARCH FOUNDATION (US)) 29 January 1998 (1998-01-29) page 5, line 12 -page 17, line 5 ---	3,7,9, 16,20, 24,26-33
X	ZHONG R. ET AL.: "Dual Methylation Pathway in Lignin Biosynthesis" PLANT CELL, vol. 10, December 1998 (1998-12), pages 2033-2045, XP002187458 page 2034, column 2, line 3 -page 2037, column 2, line 2 page 2039, column 1, line 8 -page 2041, column 2, line 29 ---	1-25,27, 31

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[X]	Further documents are listed in the continuation of box C.	[X]	Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2002	05/02/2002

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Authorized officer Schönwasser, D

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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X	WO 94 23044 A (SAMUEL ROBERTS NOBLE FOUNDATIO) 13 October 1994 (1994-10-13) page 2, line 1 - line 20 page 10, line 18 -page 13, line 3 page 17, line 19 -page 19, line 19 ---	3,7,9, 16,20, 27,31
X Y	WO 93 05160 A (ICI PLC) 18 March 1993 (1993-03-18) page 1, line 1 -page 2, line 25 page 5, line 19 - line 32 page 10, line 22 -page 12, line 28 ---	9 1-12, 14-21, 26-33
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Intc... ...onal Application No
PCT/US 01/09398

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